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REPORT DATE: June 20FG

TYPE OF REPORT: Øæ æ

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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1. REPORT DATE (DD-MM-YYYY) 01-06-2012		2. REPORT TYPE Final		3. DATES COVERED (From - To) 1 Jun 2011 - 31 May 2012
4. TITLE AND SUBTITLE Multifunctional Virus-Nanoshell Assembly for Targeted Hyperthermia and Viral Gene Therapy for Breast Cancer				5a. CONTRACT NUMBER
				5b. GRANT NUMBER W81XWH-11-1-0297
				5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Dr. Fang Wei E-Mail: fwei@rice.edu				5d. PROJECT NUMBER
				5e. TASK NUMBER
				5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) William Marsh Rice University Houston, TX 77005				8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				
13. SUPPLEMENTARY NOTES				
14. ABSTRACT We aimed to develop a virus-Au NS assembly to specifically target breast cancer cells, and to use localized heat of NIR radiation of Au NS to destroy breast cancer cells in synergy with gene therapy. We proposed to develop virus-nanoshell assemblies by attaching adeno-associated virus (AAV) to gold nanoshells (Au NS) through chemical bonds. We have successfully completed majority of tasks 1 and 2 of our Statement of Work. Specifically, we have designed and synthesized a linker molecule with dual functionalities to be able to covalently attach AAV to Au NS surface. Au NS was successfully functionalized with the linker molecules. AAV capsid surface was modified with a benzaldehyde functional group. Using higher titer AAV8, we were able to confirm and quantify the successful conjugation. Recombinant AAV vectors encoding a gene under regulation of a heat-responsive promoter were generated. By combining breast cancer targeting, gene therapy, and hyperthermia, the virus-NS assemblies have the potential to greatly reduce the recurrence of cancer, reduce side effects, increase patient survival rates, and improve the quality of life of breast cancer patients.				
15. SUBJECT TERMS Hyperthermia, Gene therapy, virus, Au nanoshell				
16. SECURITY CLASSIFICATION OF:		17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 9	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U			c. THIS PAGE U

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INTRODUCTION

Combination of hyperthermia with gene therapy may be a promising approach for breast cancer treatment. Previous work by others has shown that synergistic effects of hyperthermia-induced gene therapy increase the effects of hyperthermia at both the cellular and systemic levels. Moreover, viral gene delivery vectors delivering cargo driven by various heat-inducible promoters show positive anti-tumor effects with the combination of hyperthermia and gene therapy. We proposed to develop virus-nanoshell assemblies by attaching adeno-associated virus (AAV) to gold nanoshells (Au NS) through chemical bonds. AAV is currently one of the most promising and extensively investigated viral vectors for gene therapy. Au NSs are nanoparticles consisting of a dielectric core surrounded by a thin layer of Au. The unique property of Au NS is the tunability of its plasma resonance. Au NS can accumulate heat upon irradiation with NIR light, which is very useful for biomedical applications because tissues are transparent to NIR. Using NIR irradiation, the Au NS can be induced to generate localized heat in tissues. The generated heat will then activate the expression of delivered suicide genes that are under the regulation of heat-inducible promoters. Synergistic effects of hyperthermia and delivered genes should increase the specific and effective killing of breast cancer cells.

BODY

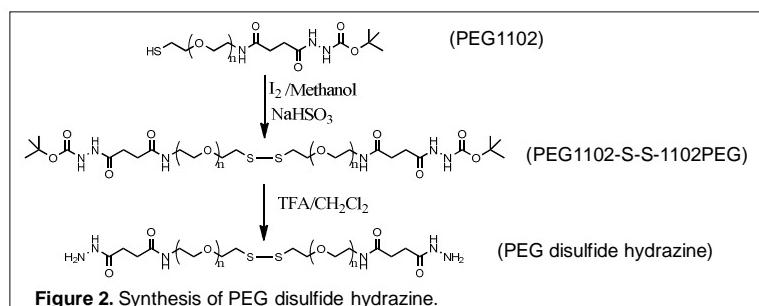
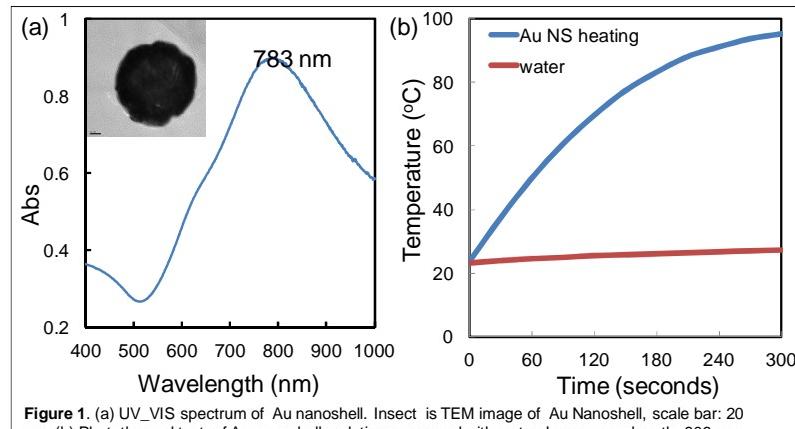
TASK #1: Generate virus NS assemblies

Au NS was synthesized via the following methods. SiO_2 was functionalized with 3-aminopropyltrimethoxysilane (APTMS). The Au colloid was synthesized following Duff method. The functionalized SiO_2 was mixed with Au colloid to form a “seed” with approximately a 30% surface coverage of gold nanoparticles on the SiO_2 surface. The seed solution was mixed with HAuCl_4 , and formaldehyde was added as a reducing agent to form a complete gold layer on the SiO_2 . TEM was used to confirm the size and completeness of the Au shell formation on the nanoparticles. UV-VIS was used to evaluate the plasmon resonance. Shown in Figure 1 (a)

insert is the TEM image of the Au NS. The size of the Au NS is about 164 nm in diameter. Shown in Figure 1 (a) is the UV-VIS spectrum of a batch of complete Au NS; the peak at 783 nm is the plasmon resonance of the Au NS. The narrowness of the peak indicates it has a complete Au layer on the surface. The photothermal capability of Au NS was tested by using 600 μl Au NS solutions with OD (optical density) of 1.4 and absorbance of 783 nm. The solution was heated with 806 nm laser of 4W power and compared with 600 μl water under the same experimental condition (Figure 1 (b)). In about 5 minutes, the Au NS solution was almost boiling but the temperature of the water only sample increased a few degrees.

Linker molecule polyethylene glycol (PEG) disulfide hydrazine was designed to use the disulfide or thiol group to form Au-S bonds to covalently attach the molecule on Au NS surface. Hydrazine group was used to form hydrazone bond with aldehyde functional group on adeno-associated virus (AAV). Hydrazone bond is stable in physiological condition and labile in acidic condition.

PEG disulfide hydrazine was synthesized using the scheme in Figure 2. First, commercially available PEG1102 was oxidized with iodine in methanol. The reaction mixture was purified with dialysis. Figure 3 shows the MALDI-MS of the starting material PEG1102 and oxidized PEG1102SSPEG1102. The expected mass of starting material is about 3000 Dalton and 6000 Dalton for the disulfide compound. The average mass obtained for the starting is 3121.6 Dalton and 6161.5 Dalton for the product.



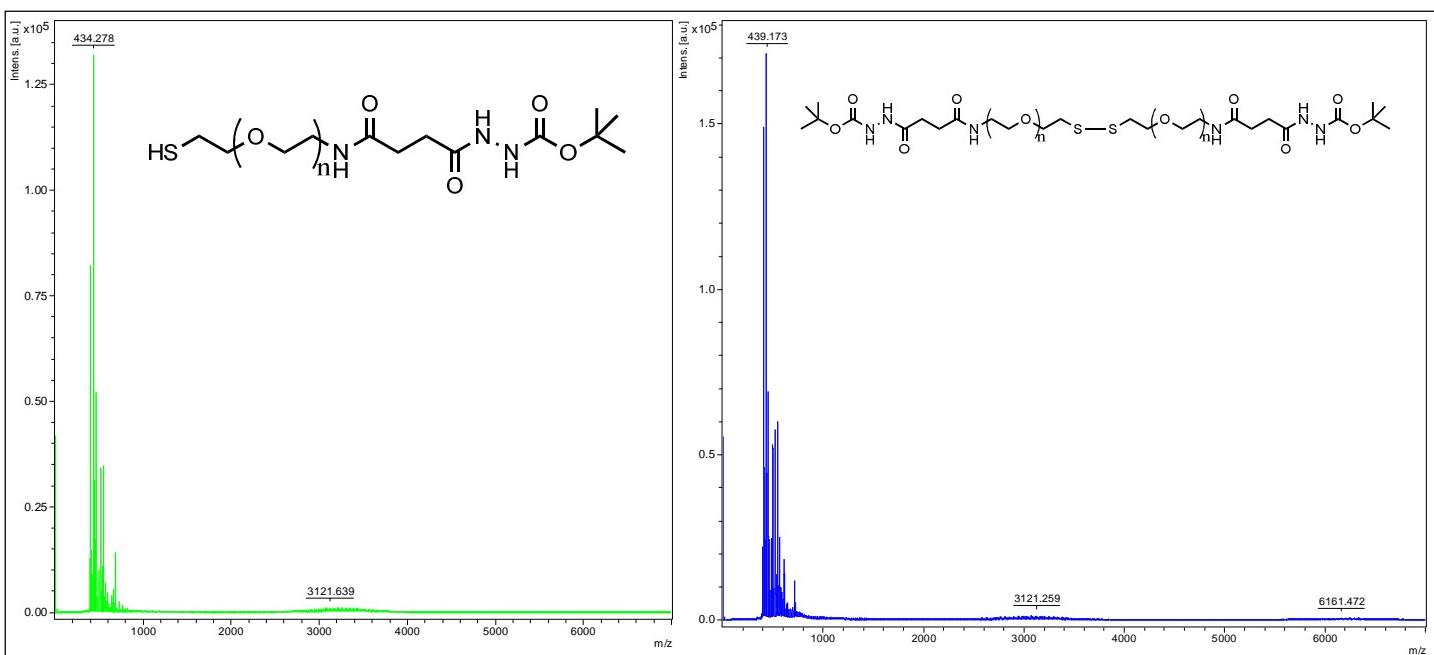


Figure 3. Mass spectra of PEG1102 and PEG1102-S-S-1102PEG .

Next, the PEG 1102 disulfide with ter-Butyloxycarbonyl (BOC) protection group was reacted with trifluoroacetic acid (TFA) in dichloromethane to remove the BOC group. The successful deprotection was confirmed by proton NMR as shown in Figure 4. The blue spectrum is the starting material (PEG1102), the red spectrum is disulfide PEG with BOC protection group (PEG1102-S-S-1102PEG). Both of them show an intense peak at 1.5 ppm, which is the characteristic peak of proton on t-butyl group. The green spectrum is the deprotected product (PEG disulfide hydrazine); it showed a complete removal of the peak at 1.5 ppm, which suggested the successful deprotection of BOC group and formation of PEG disulfide hydrazine.

Next, 900 μ l of 1mM deprotected disulfide PEG hydrazine was added into 5ml Au NS solution (about 1e9 particles/ml), protected from light, and rocked overnight at room temperature. The functionalized Au NS was purified by centrifugation with 500g for 5 minutes for three times. The synthesis scheme is shown in Figure 5.

To confirm the successful functionalization of Au NS, two tests were performed. First, we tested the stability of the functionalized Au NS by mixing Au NS solution with 100 mM NaCl and compared it with a negative control (Au NS only) and a positive control (Au NS with 100 mM NaCl). UV-Vis spectra for the three samples were collected every 10 minutes for 4 hours as shown in Figure 6. The positive control of Au NS with salt showed the absorbance of the Au NS decreased in the four hour period and the NSs were destroyed. In

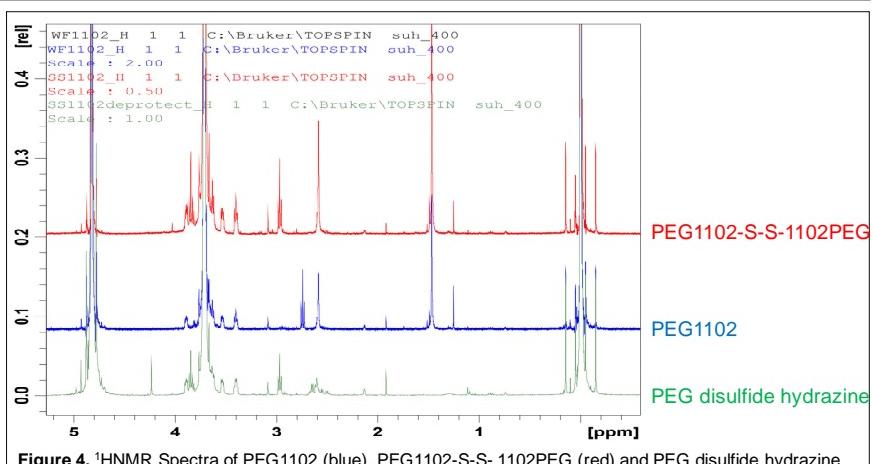


Figure 4. ^1H NMR Spectra of PEG1102 (blue), PEG1102-S-S-1102PEG (red) and PEG disulfide hydrazine (green) .

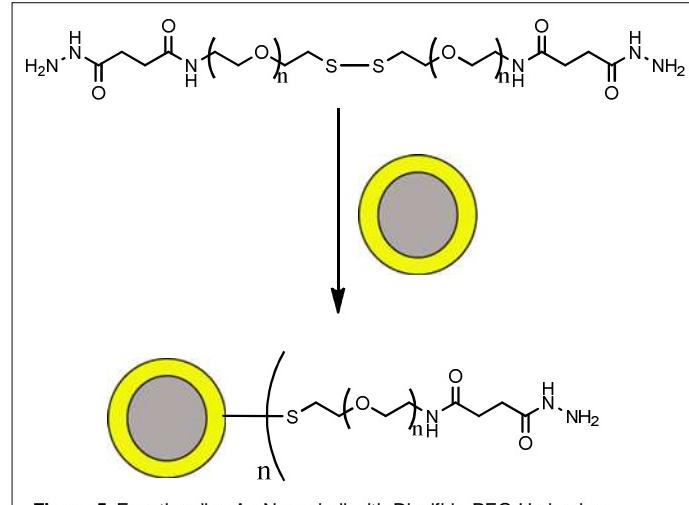


Figure 5. Functionalize Au Nanoshell with Disulfide PEG Hydrazine.

contrast, the PEG protected NS remained stable over time. It is noteworthy that the absorbance peak of the NS had a slight red shift from 788 nm to 791 nm after functionalization, which is another indication of our successful chemical conjugation of Au NS with PEG hydrazine. Another test we performed was using Dynamic Light

Scattering (DLS) to confirm the hydrodynamic size of the functionalized Au NS. Au NS has a size of 164 nm; PEG-Au NS is 191 nm, which is 27 nm increases in diameter. It is about the size of the PEG chain, but it also depends on the conformation of the PEG chain on the NS surface.

Our previous study showed that there are over 1,000 lysine residues on the surface of AAV. Among them, 300 are solvent accessible. By using NHS chemistry, the amine groups of lysine residues on AAV capsid can react with sulfo-S-4FB in PBS buffer to form amide bonds and covalently attach 4FB to the AAV capsid surface as shown in Figure 7. The product (AAV-4FB) has a terminal benzaldehyde group which later on will react with the linker molecule to form hydrazone bonds. The successful conjugation can be confirmed by reacting AAV-4FB with 2-hydraxinopyridine as shown in Figure 8(a). 2-hydraxinopyridine reacts with AAV-4FB to form the compound which absorbs at 350 nm. The molar substitution ratio (MSR) can be estimated by using a colorimetric reaction and calculated to be 108 lysines on

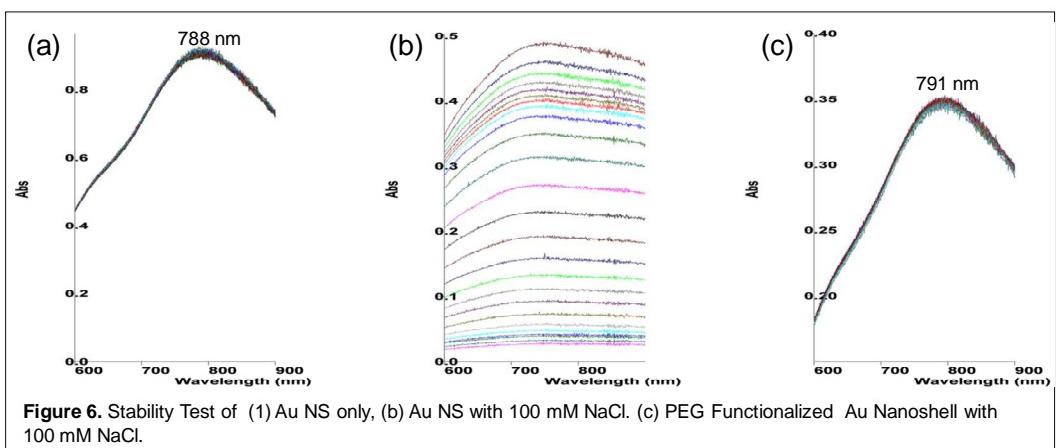


Figure 6. Stability Test of (1) Au NS only, (2) Au NS with 100 mM NaCl. (3) PEG Functionalized Au Nanoshell with 100 mM NaCl.

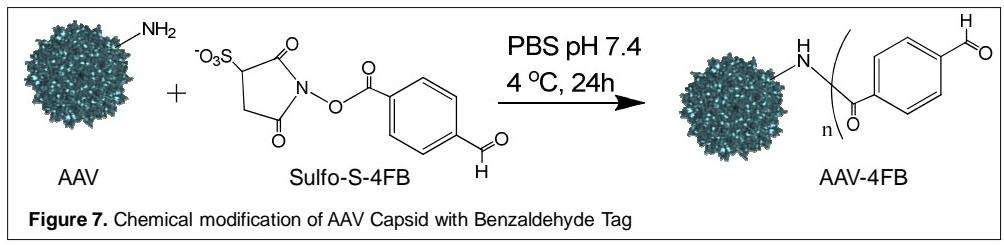


Figure 7. Chemical modification of AAV Capsid with Benzaldehyde Tag

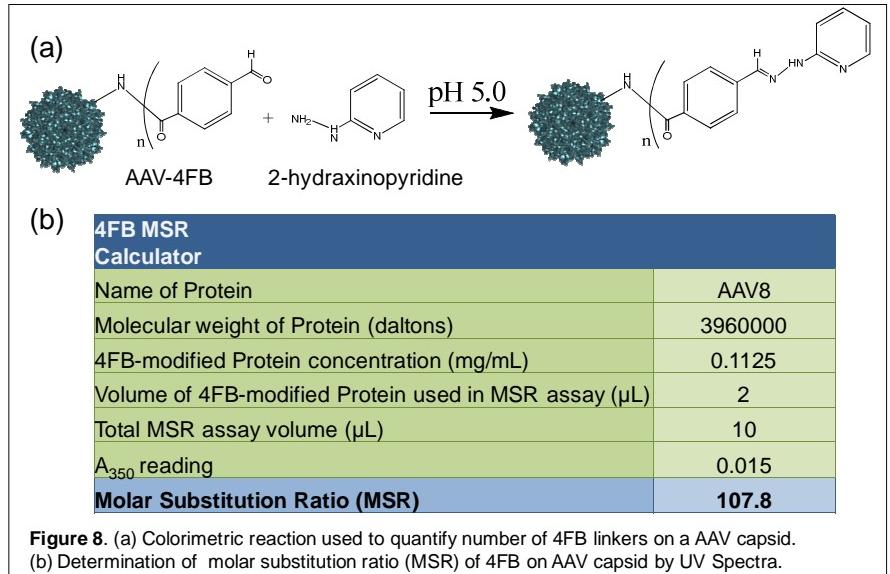


Figure 8. (a) Colorimetric reaction used to quantify number of 4FB linkers on a AAV capsid.
(b) Determination of molar substitution ratio (MSR) of 4FB on AAV capsid by UV Spectra.

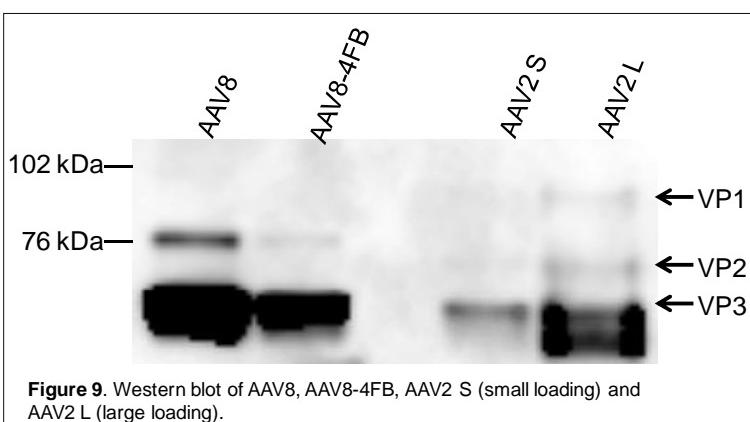


Figure 9. Western blot of AAV8, AAV8-4FB, AAV2 S (small loading) and AAV2 L (large loading).

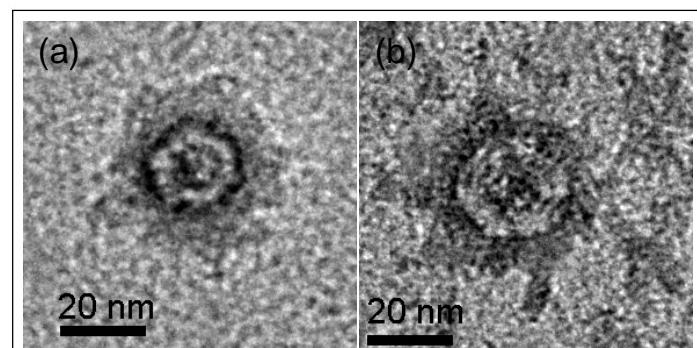


Figure 10. TEM image of (a) AAV8 and (b) AAV8-4FB. Images were negatively stained with uranyl formate.

each capsid as shown in Figure 8 (b).

Since our lab mainly works on serotype AAV2, we had difficulty obtaining higher titer virus which prevented our ability to quantify amount of functional groups on AAV capsid. Toward the end of the project we were able to obtain higher titer AAV8 from our collaborator, which enabled us to quantify the MSR and continue the planned studies.

We performed western blot of AAV8, conjugated AAV8-4FB and compared them with AAV2 as shown in Figure 9. We found that after conjugation, the capsid protein VP2 and VP3 did not change. The conjugation AAV8-4FB has lower concentration of virus due to reaction and dialysis; the signals are not as strong as AAV8. We also used TEM to confirm the integrity of the virus capsid after conjugation as shown in Figure 10. We found that after conjugation, the size of the virus somewhat increased, but the capsid is still assembled.

Several attempts to synthesize AAV NS assembly under acidic conditions were not successful. We found Au NS is not stable under pH 5.0. Some studies have shown that the hydrazone formation reaction can also occur under basic conditions. We tested this by using two different buffers, sodium acetate solution (pH 7.2) and potassium carbonate solution (pH 10.0). 0.5 mM 2-hydraxinopyridine in 100mM sodium acetate or potassium carbonate solutions was made and 1mg/ml Sulfo-S-4FB was added. Then the solutions were incubated at 37 degrees for 30 minutes. Each sample was done in duplicate. The UV spectra were obtained as shown in Figure 11. Me₂S buffer was used as a positive control - it shows an obvious peak at 350 nm which is the indication of hydrazone bond formation. In NaAc buffer at pH 7.2, even though the peak shifted to 342 nm, it is still a large peak. In K₂CO₃, the hydrazone bond formation was not successful. It appears if we need to have hydrazone bond formation in more basic conditions, NaAc would be a good choice.

TASK #2. Engineer AAV to target breast cancer cells

2c. Generate AAV vectors encoding heat-responsive promoters (HT-Prom) & therapeutic genes for heat-responsive gene therapy.

HSP70Bp Extraction from pGL4-HSP70B-Luc

Routine PCR was performed with two different primer sets (Table 1) in order to establish NcoI and BamHI restriction sites at either end of the HSP70Bp for an oligonucleotide annealing strategy (Figure 12). The resulting two PCR reaction mixes were treated with DPN1, combined, and annealed to construct the sticky- ended HSP70Bp using a set temperature of 95°C for 4 minutes followed by temperature ramp decrease to 25°C over about 1

hour on a heat block. Annealing was carried out in TNE buffer (10 mM Tris, pH 7.5-8.0, 50 mM NaCl, 1 mM EDTA). The desired annealed product contained 5' sticky ends: a BamHI site on the upstream 5' end and an NcoI site on the downstream 3' end of the promoter, which includes a Kozak sequence directly upstream of the NcoI site, a sequence necessary for efficient translation in eukaryotic cells.¹ All products from the annealing procedure were also phosphorylated on the 5' ends using T4 Polynucleotide Kinase (New England Biolabs) to ensure phosphorylated sticky ends. Sticky end verification was performed by ligating the insert to itself in a concatemer test (Figure 13). The resulting HSP70Bp insert were ligated to the pAAV-GFP backbone vector.

Restriction Digest of pAAV-CMVp-GFP

The pAAV-GFP vector backbone was prepared by performing a double digest with NcoI and BamHI on pAAV-CMVp-GFP to remove the CMVp along with the Kozak sequence while retaining GFP. The digest also

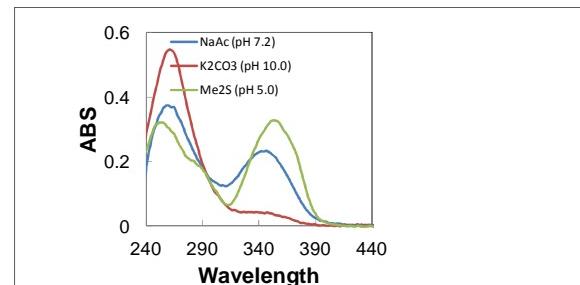


Figure 11. UV-Vis spectra of hydrazone bond formation reaction in different buffers. NaAc (pH 7.2, Blue), K₂CO₃ (pH 10.0, red) and Me₂S (pH 5.0, green).

Table 1: Primer sets used to subclone HSP70Bp from pGL4-HSP70Bp-Luc	
HSP70Bp Primers (5' → 3')	
PCR1	
HSP70B' Pro-FW1	GATCCAGAGCCAGCCGGGAGGGAGCTAGAAC
HSP70B' Pro-RV1	GTGGCGGCAGCTTGTGCGATGCTGGAGGC
PCR2	
HSP70B' Pro-FW2	CAGAGCCAGCCGGGAGGGAGCTAGAAC
HSP70B' Pro-RV2	CATGGTGGCGGCAGCTTGTGGATGCTGGAGGC

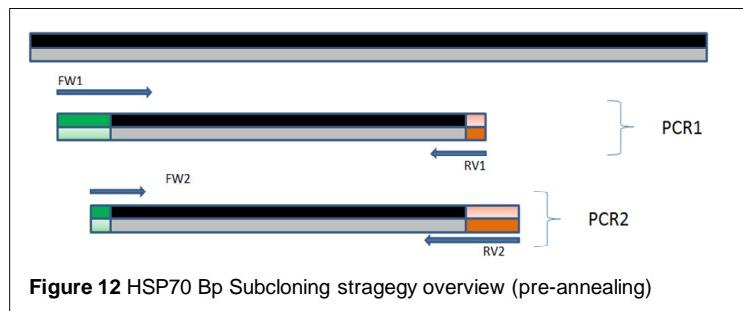


Figure 12 HSP70 Bp Subcloning strategy overview (pre-annealing)

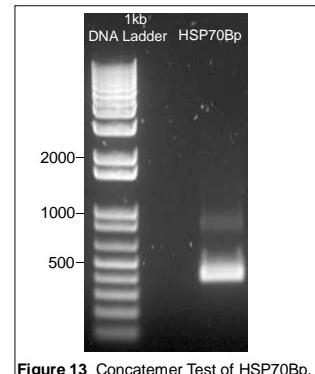


Figure 13 Concatemer Test of HSP70Bp.

removed the beta globin promoter and human beta globin fragments which were considered unnecessary for successful gene expression of GFP. The vector backbone was gel purified and extracted. The expected backbone size was ~ 6 kb along with three smaller fragments as a result of internal Ncol and BamHI restriction sites in CMVp and Beta globin promoter, respectively (Figures 14 and 15). The large ~6000 kb fragment with the other 3 smaller fragments denotes a successful digestion where largest band was extracted for purification and ligation with the HSP70Bp insert. The resulting vector was treated with calf intestinal phosphatase (New England Biolabs) to prevent re-ligation of the vector backbone.

Construction of pAAV-HSP70Bp-GFP

The resulting pAAV-GFP vector backbone and the HSP70Bp insert were ligated together using a standard ligation reaction with T4 DNA ligase in ligase buffer. A 1:6 and 1:0 (positive control) vector-to-insert ratio were used to prepare the construct for transformation. The resulting ligation reactions were transformed into DH5 α E.coli cells using heat shock (42C for 45 sec).

Colony Screening

Eight colonies were selected and two were sequenced. The sequencing overlap results for the desired plasmid are shown in Figure 16. The overlapping sequence matched perfectly for the region from the beginning of the HSP70B promoter, at the BamHI site, until midway into the GFP where a constructed sequencing primer attached. Base pair substitutions and insertions were detected upstream of the HSP70B promoter, but should not affect the overall effectiveness of the heat shock response.

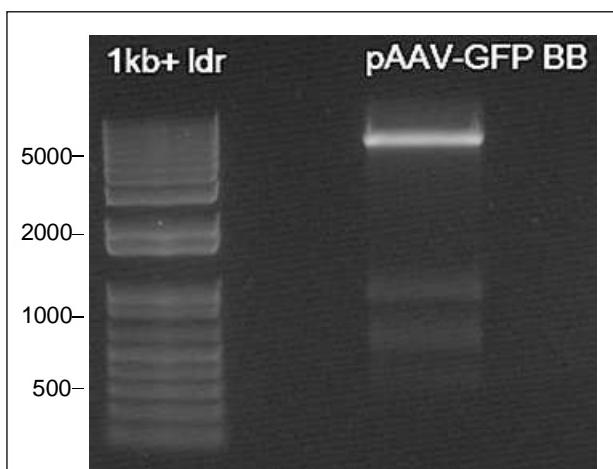


Figure 14 Gel Extraction and Purification of pAAV-GFP vector backbone (BB).

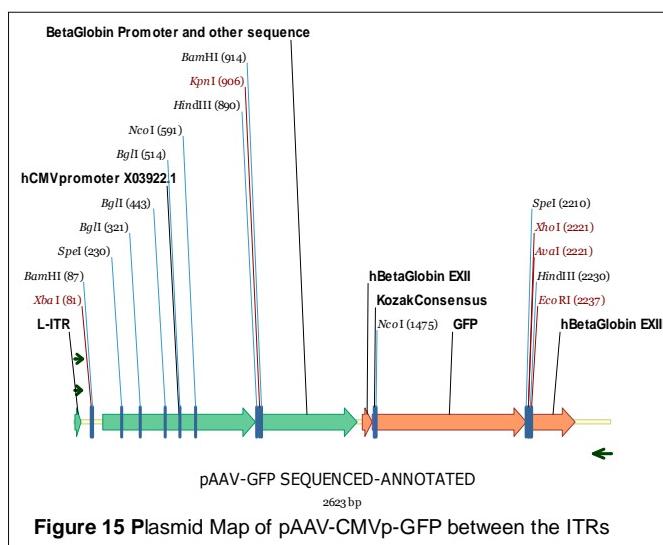


Figure 15 Plasmid Map of pAAV-CMVp-GFP between the ITRs

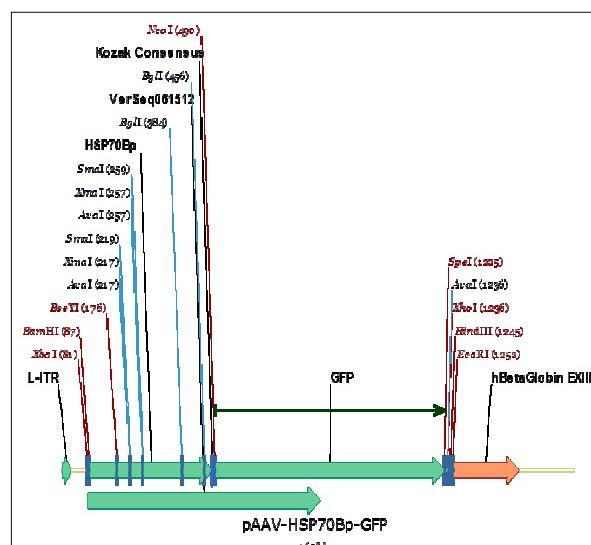


Figure 16 pAAV-HSP70Bp-GFP plasmid map. The lower green feature represents the sequencing results of the desired plasmid and where the complementary overlapping begins and ends.

KEY RESEARCH ACCOMPLISHMENTS

- Designed and synthesized linker molecule for the AAV NS assembly, purified the product and confirmed the product by MALDI-MS and ^1H NMR.
 - Functionalized Au NS with linker molecule confirmed the product with stability test and DLS measurement.
 - Conjugated 4FB molecules to AAV capsid surface through amide bonds. Conjugation was confirmed and quantified by UV-VIS spectroscopy. Western blot using B1 antibody and TEM confirmed the integrity of the conjugation.
 - Tested the experimental condition for functionalized Au NS and AAV hydrazone formation. Sodium acetate

solution of pH 7.2 would be a good choice.

- Designed and constructed recombinant AAV encoding GFP driven by heat-inducible promoter. Colony screening shows successful insertion of HSP promoter into the construct.

REPORTABLE OUTCOMES

Presentations

- "Gene Therapy with Hyperthermia for Targeted Breast Cancer Treatment Using Virus-Nanoshell Assembly" oral presentation at 28th Southern Biomedical Engineering Conference, Houston, Texas, May 6, 2012.

CONCLUSION

We have successfully completed majority of tasks 1 and 2 of our Statement of Work. Specifically, we have designed and synthesized a linker molecule with dual functionalities to be able to covalently attach AAV to Au NS surface. Au NS was successfully functionalized with the linker molecules. AAV capsid surface was modified with a benzaldehyde functional group. Due to difficulties with low virus titers, we had to switch from AAV2 to AAV8 towards the latter part of our project. Using higher titer AAV8, we were able to confirm and quantify the successful conjugation. Recombinant AAV vectors encoding a gene under regulation of a heat-responsive promoter were generated. By combining breast cancer targeting, gene therapy, and hyperthermia, the virus-NS assemblies have the potential to greatly reduce the recurrence of cancer, reduce side effects, increase patient survival rates, and improve the quality of life of breast cancer patients.

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